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TITLE: **Governance of Cutaneous Photocarcinogenesis by Chronic UVA-Exposed Dermal Fibroblasts**

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Long-wave ultraviolet A (UVA) is the major component of terrestrial UV radiation and is also the predominant constituent of indoor sunlamps, both of which have been shown to increase cutaneous melanoma risk. Using a 2-chamber model, we show that UVA-exposed target cells induce an intercellular oxidative signaling to non-irradiated bystander cells. This UVA-mediated bystander stress is observed between all three cutaneous cell types (i.e. keratinocytes, melanocytes and fibroblasts). Significantly, melanocytes appear to be more resistant to direct UVA effects compared to keratinocytes and fibroblasts although melanocytes are also more susceptible to bystander oxidative signaling. The extensive intercellular flux of oxidative species has not been previously appreciated and could possibly contribute to the observed cancer risk associated with prolonged UVA exposure, such as those associated with indoor tanning.

**15. SUBJECT TERMS**

Carcinogenesis, melanoma, ultraviolet radiation

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**AWARD NUMBER:** W81XWH-10-1-0436
**TITLE:** Governance of cutaneous photocarcinogenesis by chronic UVA-exposed dermal fibroblasts
**PRINCIPAL INVESTIGATOR**: Hensin Tsao, MD PhD

## 1. INTRODUCTION

Long-wave ultraviolet A (UVA) is the major component of terrestrial UV radiation and is also the predominant constituent of indoor sunlamps, both of which have been shown to increase cutaneous melanoma risk. Using a 2-chamber model, we show that UVA-exposed target cells induce an intercellular oxidative signaling to non-irradiated bystander cells. This UVA-mediated bystander stress is observed between all three cutaneous cell types (i.e. keratinocytes, melanocytes and fibroblasts). Significantly, melanocytes appear to be more resistant to direct UVA effects compared to keratinocytes and fibroblasts although melanocytes are also more susceptible to bystander oxidative signaling. The extensive intercellular flux of oxidative species has not been previously appreciated and could possibly contribute to the observed cancer risk associated with prolonged UVA exposure, such as those associated with indoor tanning.

## 2. KEYWORDS

Melanoma, melanocytes, UVA, radiation, bystander, reactive oxygen species, oxidative stress

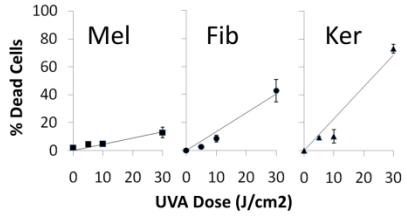
## 3. OVERALL PROJECT SUMMARY

Results are outlined and associated **Tasks** are in **bold**

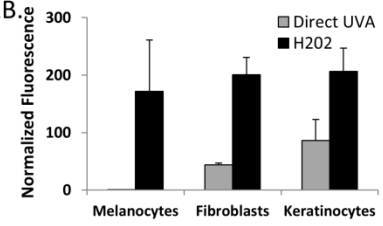
*Aim 1. Establish the extent of melanocyte and keratinocyte bystander genotoxicity (i.e. DNA and cellular) from UVA-targeted fibroblasts and determine if bystander oxidative stress is induced by UVA irradiation and abrogated by antioxidants.*

In initial studies, we selected primary human skin cells in order to avoid untoward effects of immortalization or transformation. Initial experiments determined direct effects of UVA on normal human melanocytes (NHMs), fibroblasts (NHFs) and keratinocytes (NHKs). Figure 1A shows dose-dependent toxicity for three skin cell types exposed to UVA under identical conditions of illumination and cell density (**Task 1.1b, 1.1c**). Among these cell types, pigmented NHMs appeared to be least susceptible to direct UVA toxicity. Figure 1B depicts the levels of oxidative stress (measured using the reactive oxygen species (ROS) probe, CMDHDCF) generated in each cell type as a function of UVA illumination (10 J/cm$^2$)

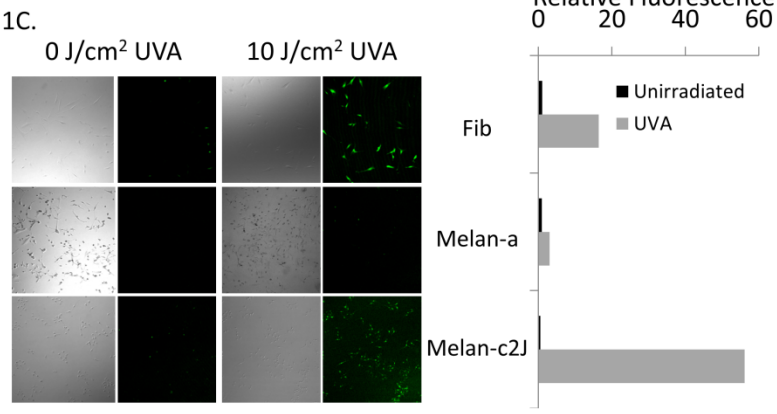
1A.




1B.

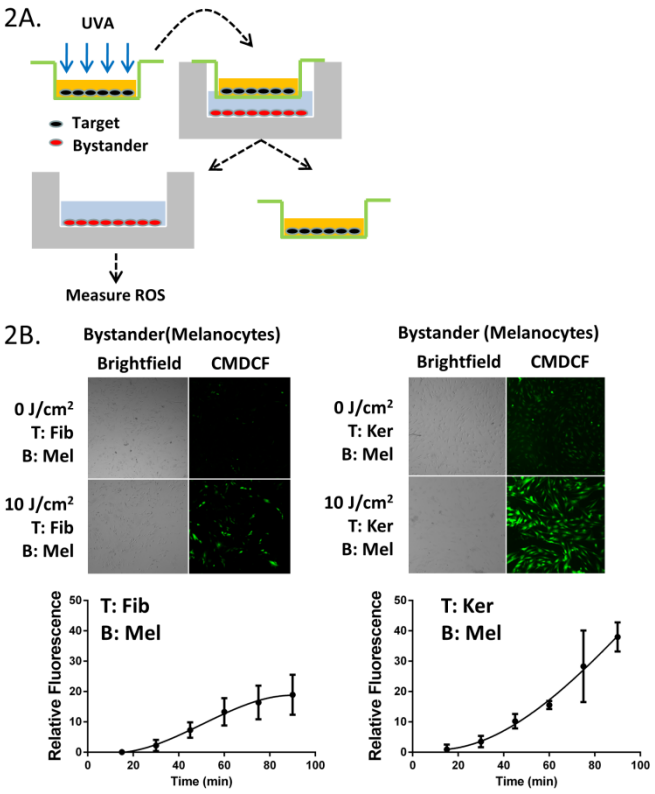



1C.



with $H_2O_2$ as a positive oxidative control (**Task 1.1d, 1.1e**).

Consonant with viability, melanocytes also generated lower amounts of ROS on direct UVA illumination although all three cell types were responsive to extracellular $H_2O_2$. This suggests that the presence of melanin may in fact protect against the effects of direct UVA exposure. To explore this possibility, we subjected immortalized C57BL6 melanocytes from normal (i.e. melan-a) and albino (i.e. melan-c2J) backgrounds to direct UVA irradiation (10 J/cm$^2$). Figure 1C shows that loss of melanin in the melan-c2J cells was associated with an increase in the level of ROS compared to the eumelanized melan-a cells. Taken together, these results reveal that human skin cells undergo oxidative stress upon UVA exposure and that pigmented melanocytes are more resistant to the direct effects of UVA compared to other skin cells. It also raises the possibility that intercellular flux of ROS between normal human cells may represent a previously unappreciated source of stress signaling.




To better characterize the collateral oxidative signaling induced by UVA irradiation, we created an interchangeable 2-compartment model (**Task 1.1a**; Fig 2A) to manipulate and to quantify bystander stress. As bystander effects are more pronounced at low fluence where damage occurs but cell killing is low(Chakraborty et al., 2009), a fluence of 10 J/cm$^2$ UVA was chosen for all subsequent studies. In initial analyses, unirradiated melanocytes (i.e. bystander) that were co-cultured with either UVA-treated NHKs or NHFs (i.e. the targets) exhibited a consistent time-dependent increase in DCF fluorescence (**Task 1.2a, 1.2b**; Fig 2B). In contrast, bystander melanocytes co-cultured with unirradiated cells led to no appreciable induction of DCF fluorescence.

Experiments were then performed using all combinations of target and bystander populations to determine which cell types are receptive to bystander signaling and which cell types are efficient at generating signal when treated with UVA. Table 1 shows the corrected DCF fluorescence signal in the bystander wells after 90 minutes of co-culture with UVA-illuminated target

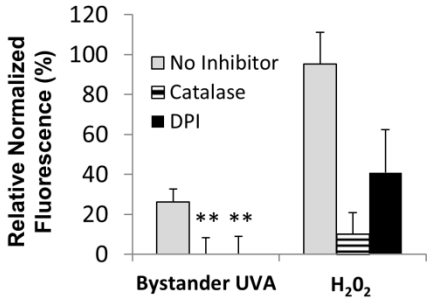
**Table 1. Cell-cell UVA Bystander Stress Signaling**

| | | UVA Target (ROS Donor) | | | |
|---|---|---|---|---|---|
| | | Keratinocyte | Melanocyte | Fibroblast | *Mean "recipient" index‡* |
| Bystander (ROS recipient) | Keratinocyte | 20.4# ± 5.4 (3)* | 13.5 ± 0.9 (4) | 0 ± 6.4 (3) | *11.3* |
| | Melanocyte | 49.0 ± 0.5 (8) | 15.4 ± 3.5 (5) | 29.5 ± 6.7 (4) | *31.3* |
| | Fibroblast | 31.3 ± 1.4 (6) | 0 ± 7.2 (4) | 11.7 ± 4.7 (4) | *14.3* |
| | *Mean "donor" index†* | *33.6* | *9.6* | *13.7* | |

# To compare across experiments, we normalized UVA bystander stress to maximum observed with 200 μM $H_2O_2$
corrected stress = 100*($DCF_{10J}$-$DCF_{0J}$)/$DCF_{H2O2}$
* number of experiments shown in parentheses
† mean corrected stress among all recipients due to specific donor cell type (ie. average of column values)
‡ mean corrected stress experienced by a single recipient cell type (ie. average of row values)

populations. As an ROS donor, keratinocytes appeared to generate the greatest amount of bystander stress upon UVA illumination. The greatest effect was in fact observed when keratinocytes and melanocytes were paired as target and bystander (corrected stress = 49.0 + 0.5), respectively. Similar results were obtained with two different sources of primary melanocytes. Overall, melanocytes had the highest mean "recipient" index compared to other cells (32.5 vs 11.3 and 14.3) suggesting that pigment cells experience the greatest bystander stress irrespective of which target cell type was treated with UVA (**Task 1.2c**). Interestingly, melanocytes were the least effective donors of stress signaling when irradiated as targets, consistent with the lower response upon direct UVA exposure (Fig 1). Keratinocytes appeared to be most resistant to bystander stress while fibroblasts showed intermediate efficiencies as both donor and recipient.

3A.

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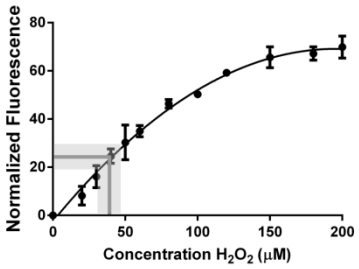
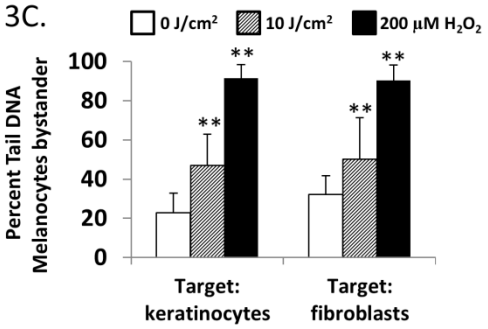



We next performed experiments to gain greater insight into the nature and extent of the signaling event. When the target fibroblasts were pre-treated with either extracellular catalase (a $H_2O_2$ scavenger) or diphenylene iodinium (DPI, an NADPH oxidase inhibitor), complete abrogation of DCF fluorescence in the melanocytes was observed (**Task 1.2d**; Fig 3A). These results document intercellular transmission of $H_2O_2$, and possibly other species, to bystander melanocytes upon UVA irradiation of co-cultured fibroblasts. In order to approximate the level of oxidative signaling, we generated a standard curve of DCF fluorescence based on amounts of direct $H_2O_2$ exposure (Fig 3B). Since the overall normalized melanocyte bystander DCF fluorescence (N=16 bystander determinations) was 23.02±9.3, it appears that bystander oxidative stress approaches approximately 40 μM peroxide-equivalents of ROS. Furthermore, as shown in Fig 3C, both direct UVA and bystander stress signaling caused DNA damage as measured by the comet assay.

3C.




*Aim 2. Assess for mutations of TP53, CDKN2A and RAS pathway genes within the fibroblast compartment of human skin cancer specimens.*

This Aim was originally designed to look for p53 mutations, among other genes, in cells from chronically sun-damaged skin. Despite extensive optimization, the amount of cells and genetic content obtained from pilot material was insufficient for a convincing result. We thus decided to approach this question in a different way. We reasoned that if p53 was involved mechanistically, then inducing p53 through pharmacologic means should attenuate the bystander UVA stress. In order

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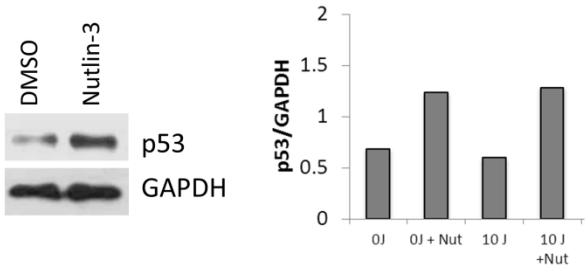
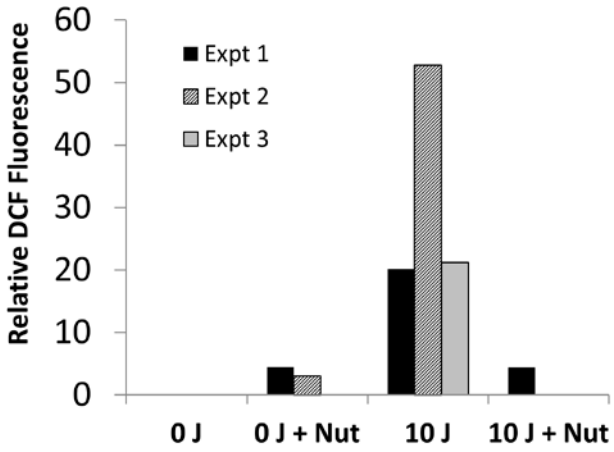


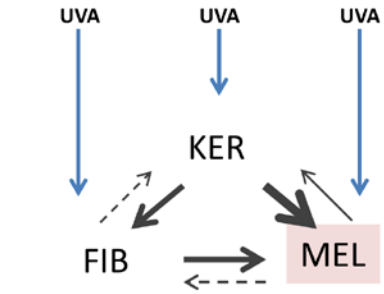
to activate p53, we used the MDM2 antagonist nutlin-3, which is known to interrupt p53-MDM2 binding and to rescue p53 from proteasomal condemnation. As shown in Figure 4, pretreatment of bystander melanocytes with nutlin-3, an MDM2 antagonist, increased cellular levels of p53 in NHMs. Strikingly, with induction of p53, there was a complete abrogation of bystander oxidative signaling in the melanocytes when co-cultured with UVA-targeted fibroblasts (Fig 5). The cost of performing the sequencing has been returned to the Dept of Defense.

5.




These initial studies support a model whereby melanocytes appear to be more resistant to direct UVA oxidative stress and the least efficient generator of bystander signaling while they were also paradoxically the most vulnerable recipients of bystander stress (Fig 6).

6.




Methodological details of the aforementioned experiments can be found in the published article,
**J Invest Dermatol. 2014 Apr;134(4):1083-90**, which is also appended.

*Aim 3. Compare the tumorigenicity of dermal melanoma xenografts in pre-solar simulated UV (SSUV) irradiated and non-irradiated athymic Nu/Nu nude mice.*

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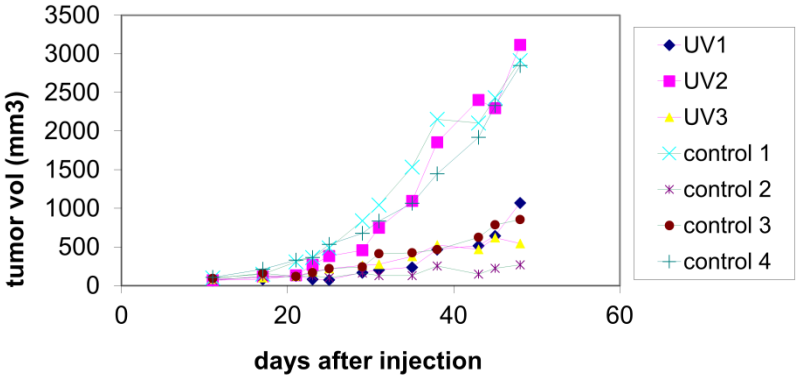


In order to determine if whole animal UVA exposure, we exposed immunocompromised (*nu*/*nu*) mice to UVA irradiation (20 J/cm$^2$) for 9 weeks (5x/week) prior to the inoculation of 3 million A375 melanoma cells. In this xenograft model, there was early results suggesting that UVA irradiation actually *diminished* the formation of melanoma tumors rather than accelerate the production of tumors, as initially thought (Task 3.1a-e. Fig 7). Given studies were challenging in several regards. First, there were no UVA irradiation chambers that were available for animal studies. As such, we had to a UVA irradiation chamber from scratch, which significantly delayed the start of UV treatments. Second, tumor growth in early studies (data not shown) exhibited highly irregular rates of growth. These biologic

inconsistencies have now been normalized and thus studies were carried out as described. Although these results have not been published, we are hopeful that additional funding can be secured through other sources in order to complete the objective.

Another line of experimentation that is just beginning to get under way is the use of genetically-engineered mouse models (GEMMs) as described in the original statement of work. The crosses have been largely infertile and highly complex. We are beginning to obtain the requisite genotypes but have not had sufficient numbers of complete the experimental tasks.

## 4. KEY RESEARCH ACCOMPLISHMENTS

The major impact of these studies is a better understanding of skin cancer causation with an eye towards improved methods of prevention.

- UVA is not benign but incredibly stressful on the skin in oxidative terms. Using approximations from our system, we estimate that the amount of bystander stress experienced by melanocytes is roughly equivalent to 40 μM $H_2O_2$-equivalents. This is likely an underestimate since the 2-compartment model reflects oxidative flux diluted into a chamber filled with 500 μL of media. The cell-cell contiguity that actually exists in vivo would be significantly greater than the detectable amount in vitro. Furthermore, since human skin is stratified with keratinocytes resting on top of the melanocytes, the most active ROS "donors" are also the cells (i.e. keratinocytes) that come into primary contact with incoming UVA. Thus, one important biological implication of our findings is that a profound flux of near-neighbor ROS envelopes each melanocyte with every UVA exposure.
- Tanning beds are likely more dangerous than previously thought. Indoor sunlamps, which predominantly emit UVA, have been touted as "safe" given their relatively lower levels of genotoxic UVB. Our studies employed a fluence of 10 J/cm$^2$ delivered at an irradiance of 10.5 mW/cm$^2$ for a duration of approximately 16 minutes. Literature reports of sunbed characteristics cite irradiances of ~20 mW/cm$^2$ UVA delivered to the skin for a fluence of 24-36 J/ cm$^2$ in a typical 20-30 minute session- a level higher than that delivered in our experiments.
- The effect of solar UVA is more far-reaching than anticipated. The UVA irradiance at the earth's surface in several cities across the USA has been estimated to be around 2.5 mW/cm$^2$, hence, the fluence of 10 J/cm$^2$ is equivalent to just over an hour's exposure under physiological conditions. Furthermore, since sunlight itself is 90-98% UVA, sunscreens that solely absorb UVB without significantly attenuating UVA will have little impact on the levels of diffusible ROS generated by keratinocytes upon solar UVA exposure.

## 5. CONCLUSION

Chronic long-wave UVA irradiation leads to a high intercellular flux of reactive oxygen species. This level of bystander stress is likely unattenuated by the readily available UVB sunscreens. Newer generations of chemopreventive agents need to account for the bystander stress.

## 6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

**Journal publications.**

Redmond RW, Rajadurai A, Udayakumar D, Sviderskaya EV, Tsao H. J Invest Dermatol. 2014 Apr;134(4):1083-90: acknowledgement of federal support **yes**.

The JID is the #1 Dermatology journal. Since the publication is recent, there has not been time to gauge impact. However, reference to this publication has already appeared online:

1. www.photobiology.info/Widel.html
2. www.newsrx.com/newsletters/Life-Science.../2504222014775LS.html

**Presentations.**
Presentations at which elements of the study were introduced and discussed:
National
2010 "New Insights into the Genetics of Moles and Melanomas"/ Department of Dermatology Martin Luther King, Jr. Visiting Professor University of Michigan; Ann Arbor, MI
2010 "Genetics of Skin Cancer", "Melanoma Review 2010" and "Journal Watching: Pigmentation and Melanoma". American Academy of Dermatology Annual Meeting, Miami, FL
2010 "Update on Melanoma Genetics"/ 7th International Symposium on Melanoma, New York, NY
2010 "Advances in Dermatology: Melanoma"/
American Academy of Dermatology Summer Meeting, Boston, MA
2010 "Melanoma Photocarcinogenesis"/ Department of Dermatology Visiting Professor University of Utah; Salt Lake City, UT
2010 "Melanoma Genetics for Risk Assessment"/ ; American Society for Clinical Oncology Annual Meeting; Chicago, IL
2010 "Melanoma and Moles: FAQs"/ Department of Dermatology Visiting Professor SUNY Downstate, Brooklyn, NY
2011 "Genetics of Skin Cancer"; American Academy of Dermatology Annual Meeting; New Orleans, LA
2012 "Pathogenesis of BCC, SCC, and Melanoma"; American Academy of Dermatology Annual Meeting; San Diego, CA
2012 "Personalized Cancer Care in the Genomic Era"/ Department of Dermatology Visiting Professor
College of Physicians & Surgeons, Columbia University; New York, NY
2012 "Personalizing Skin Cancer Care in the Genomic Era,"/ Proctor & Gamble Lecturer University of Cincinnati; Cincinnati, OH
2012 "Update in Cutaneous Oncology"; 2012 Annual Meeting of the American Dermatologic Association; Washington, DC
2013 "Update on Hereditary Melanoma Genetics"; University of Wisconsin man's Lectures Wisconsin Dermatological Society Annual meeting; Milwaukee, WI

International
2010 "Melanoma: Genes or Environment?"; German Congress on Skin Cancer; Kiel, Germany
2010 "Melanoma Biology Unmasked"; International Meeting of the Society for Melanoma Research; Sydney, Australia
2011 "Update on Melanoma Genetics"; World Congress of Dermatology; Seoul, Korea
2011 "Melanoma and Genetics"; 91st Meeting of the British Association of Dermatologists; London, England
2014 "Update on Melanoma Genetics"; 8th Canadian Melanoma Conference; Banff, Canada

**7. INVENTIONS, PATENTS AND LICENSES**
None

**8. REPORTABLE OUTCOMES**
Although there are no commercial products that have been generated through this research, the 2-chamber model for detecting bystander UVA stress is unique and created by the laboratory. The significance of the 2-chamber irradiation platform (designated MOdular DEconstruction and

Reconstruction with aNalysis (MODERN)) can be used to identify novel biologic mechanisms and also for drug screening.

## 9. OTHER ACHIEVEMENTS

None

## 10. REFERENCES

None

## 11. APPENDICES

Reprint of Journal Investigative Dermatology article

# Melanocytes Are Selectively Vulnerable to UVA-Mediated Bystander Oxidative Signaling

Robert W. Redmond[1], Anpuchchelvi Rajadurai[1], Durga Udayakumar[2], Elena V. Sviderskaya[3] and Hensin Tsao[1]

Long-wave UVA is the major component of terrestrial UV radiation and is also the predominant constituent of indoor sunlamps, both of which have been shown to increase cutaneous melanoma risk. Using a two-chamber model, we show that UVA-exposed target cells induce intercellular oxidative signaling to non-irradiated bystander cells. This UVA-mediated bystander stress is observed between all three cutaneous cell types (i.e., keratinocytes, melanocytes, and fibroblasts). Significantly, melanocytes appear to be more resistant to direct UVA effects compared with keratinocytes and fibroblasts, although melanocytes are also more susceptible to bystander oxidative signaling. The extensive intercellular flux of oxidative species has not been previously appreciated and could possibly contribute to the observed cancer risk associated with prolonged UVA exposure.

*Journal of Investigative Dermatology* (2014) **134,** 1083–1090; doi:10.1038/jid.2013.479; published online 12 December 2013

## INTRODUCTION

The cutaneous cellular community is chronically exposed to broad-spectrum sunlight, although most of the deleterious photochemical tissue interactions result from UV radiation. The impact of UV exposure on skin cancer production has been well-established through many lines of study. Decades of epidemiologic research has unequivocally linked excessive sun exposure with an increased risk of developing both cutaneous melanoma and non-melanoma skin cancer (Berwick and Halpern, 1997; Elwood and Jopson, 1997; Tsao and Sober, 1998; Almahroos and Kurban, 2004). Perhaps, the strongest direct evidence for UV participation in skin cancer formation comes from the high enrichment for C→T transitions at dipyrimidine sites in melanomas from solar-exposed locations compared with those from acral, sun-hidden regions (Berger *et al.*, 2012; Alexandrov *et al.*, 2013). Heritable defects in the repair of UV photoproducts results in xeroderma pigmentosum (Lynch *et al.*, 1984; Kraemer *et al.*, 1987)—a condition characterized by an excessive risk for melanoma among other skin cancers. Despite the substantial weight of evidence supporting the relationship between UV radiation and cutaneous carcinogenesis, the exact light-tissue interactions that govern this process are still not fully elucidated.

A more recent line of evidence has emerged with the observed association between skin cancer risk and indoor sunlamps (Han *et al.*, 2006; Clough-Gorr *et al.*, 2008; Fears *et al.*, 2011; Boniol *et al.*, 2012). Most of the energy from sunlamps is derived from long-wave UV radiation (i.e., UVA) (Autier *et al.*, 2011; Nilsen *et al.*, 2011). UVA has been shown to trigger a shower of short-lived reactive oxygen species (von Thaler *et al.*, 2010; Noonan *et al.*, 2012), which can generate 8-oxo-7,8-dihydro-2′-deoxyguanosine (Douki *et al.*, 2003) species and G→T transversions (Kozmin *et al.*, 2005). In animals, UVA has also been shown to harbor a direct melanomagenic effect (Noonan *et al.*, 2012). Unlike the fixed positional effects of direct DNA damage, UVA–induced reactive oxygen species (ROS) can freely diffuse and therefore theoretically cause near-neighbor bystander stress. Here we use a two-chamber system to show that UVA induces a rich exchange of ROS between individual cell types resident in the cutaneous community. Our results indicate that melanocytes are selectively vulnerable to UVA–mediated bystander stress. Given the high keratinocyte-to-melanocyte ratio in normal skin, we suggest that UVA exposure initiates strong oxidative signaling that envelops cutaneous melanocytes, subjecting them to profound levels of oxidative stress.

## RESULTS

In these studies, we selected primary human skin cells in order to avoid untoward effects of immortalization or transformation. Initial experiments determined direct effects of UVA on normal human melanocytes (NHMs), normal human fibroblasts, and normal human keratinocytes. Figure 1a shows dose-dependent toxicity for three skin cell types exposed to UVA under identical conditions of illumination and cell density. Among these cell types, pigmented NHMs appeared

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*E-mail: htsao@partners.org*

*Abbreviations: DCF, dichlorofluorescein; $H_2O_2$, hydrogen peroxide; NHM, normal human melanocyte; ROS, reactive oxygen species*

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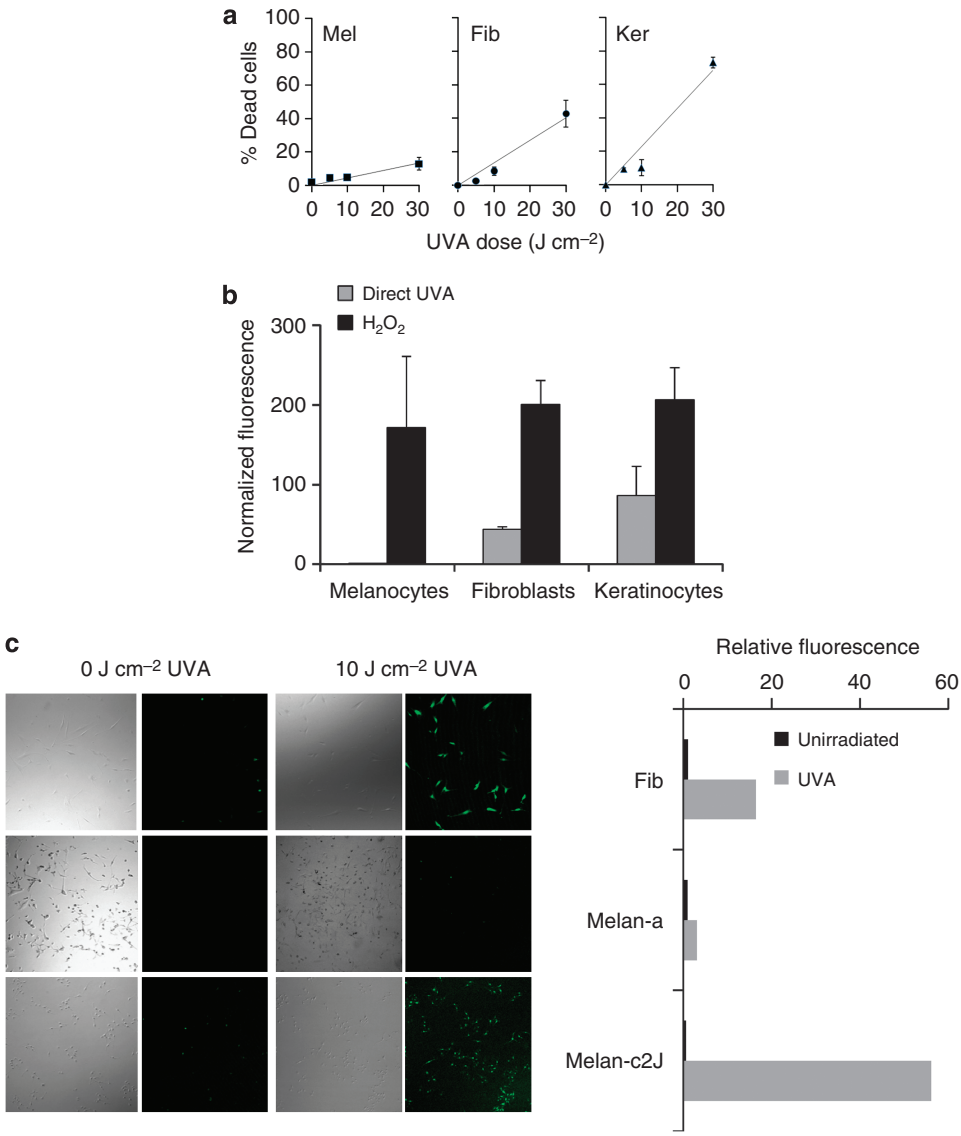


**Figure 1. Direct oxidative and toxic effects of UVA on skin cells.** (**a**) UVA induces less cytotoxicity in primary human melanocytes (Mel) compared with fibroblasts (Fib) or keratinocytes (Ker). Viability was determined by Live/Dead assay. (**b**) UVA (10 J cm$^{-2}$) elicits minimal 2′,7′-dichlorofluorescein (DCF) fluorescence in melanocytes compared with fibroblasts or keratinocytes, whereas all three cell types respond similarly to $H_2O_2$ (200 μM). Error bars represent SD within a representative experiment; all experiments were repeated two to four times. (**c**) Immortalized murine melanocytes from nonagouti/black (*a/a*) mice on a C57/BL6 background (i.e., melan-a cells) and albino ($Tyr^{c-2J}/Tyr^{c-2J}$) mice on matched C57/BL6 mice (i.e., melan-c2J cells) were subjected to UVA (10 J cm$^{-2}$); primary human fibroblasts (Fib) were used as a positive control. The level of DCF fluorescence was higher in the albino melanocytes and primary fibroblasts, suggesting that the melanin in the eumelanized melan-a cells may have mitigated the intracellular reactive oxygen species (ROS) either directly (through ROS absorption) or indirectly (through UVA absorption) or both. In the bar graph, the amount of fluorescence was normalized to the amount of fluorescence observed in unirradiated fibroblasts (set as ''1''). This experiment was repeated three times with similar results.

to be least susceptible to direct UVA toxicity. Figure 1b depicts the levels of oxidative stress (measured using the ROS probe, CM-$H_2$DCFDA) generated in each cell type as a function of UVA illumination (10 J cm$^{-2}$) with hydrogen peroxide ($H_2O_2$) as a positive oxidative control. Consonant with viability, melanocytes also generated lower amounts of ROS on direct UVA illumination, although all three cell types were responsive to extracellular $H_2O_2$. This suggests that the presence of melanin may in fact protect against the effects of direct UVA exposure. To explore this possibility, we subjected immortalized C57BL6 melanocytes from normal (i.e., melan-a) and albino (i.e., melan-c2J) backgrounds to direct UVA irradiation (10 J cm$^{-2}$). Figure 1c shows that loss of melanin in the melan-c2J cells was associated with an increase in the level of ROS compared with the eumelanized melan-a cells. Taken together, these results reveal that human skin cells undergo oxidative stress upon UVA exposure and that pigmented melanocytes are more resistant to the direct effects of UVA compared with other skin cells. It also raises the possibility that intercellular flux of ROS between normal human cells may represent a previously unappreciated source of stress signaling.

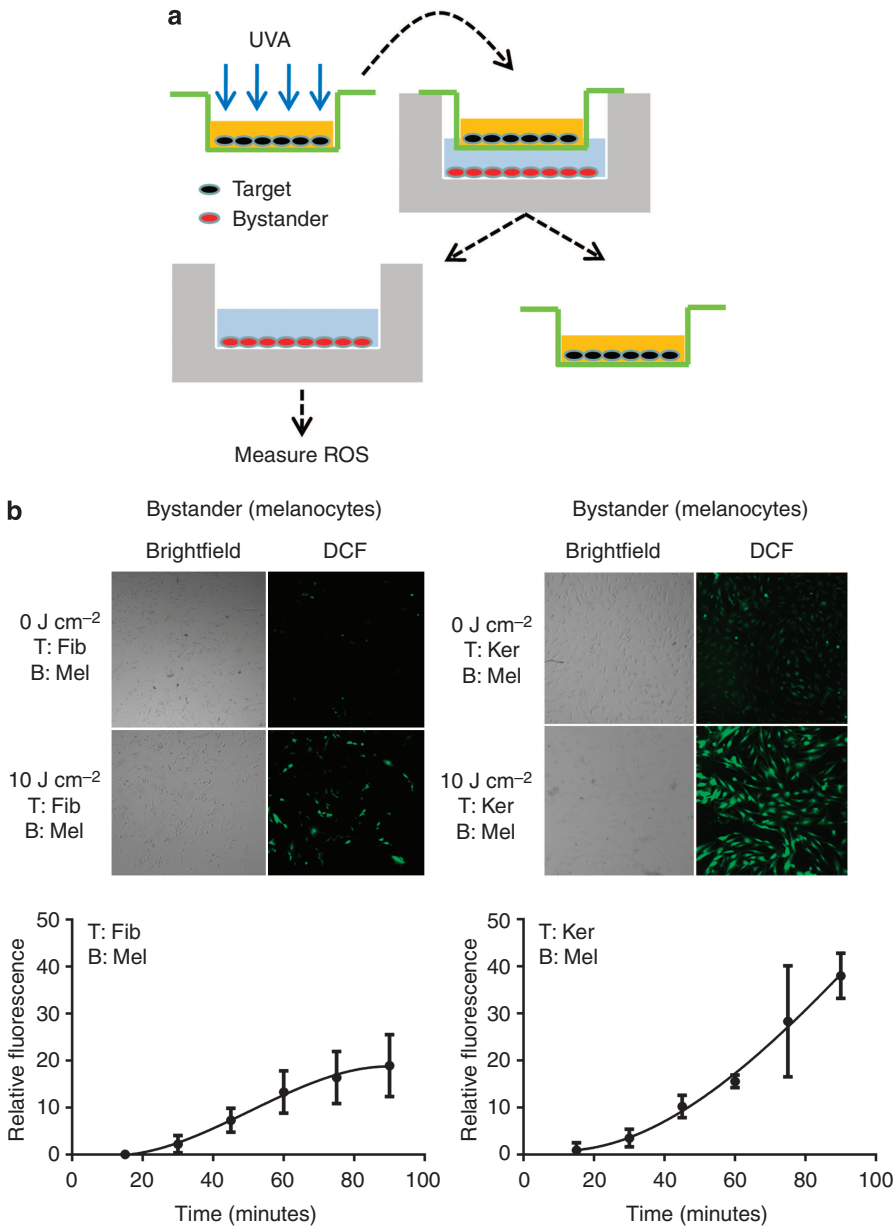


**Figure 2. UVA-induced bystander effect.** (**a**) Two-chamber (transwell/insert) model used to assess bystander stress. Wells were plated at 80,000 cells per well and inserts were plated at 40,000 cells per well. Treated cells in the inserts comprise the "target" population, whereas untreated cells in the wells comprise the "bystander" population. (**b**) Dichlorofluorescein (DCF) fluorescence in unirradiated bystander melanocytes (Mel) after 10 J cm$^{-2}$ UVA irradiation of target fibroblasts (Fib) or keratinocytes (Ker). Upper panels show DCF fluorescence by imaging, whereas lower panels show accumulation of *normalized* DCF fluorescence (DCF (10 J cm$^{-2}$) – DCF (0 J cm$^{-2}$)); thus, the graph illustrates time-dependent accumulation of UVA–mediated oxidative stress in bystander. ROS, reactive oxygen species.

To better characterize the collateral oxidative signaling induced by UVA irradiation, we created an interchangeable two-compartment model (Figure 2a) to manipulate and to quantify bystander stress. As bystander effects are more pronounced at low fluence where damage occurs but cell killing is low (Chakraborty *et al.*, 2009), a fluence of 10 J cm$^{-2}$ UVA was chosen for all subsequent studies. In initial analyses, unirradiated melanocytes (i.e., bystander) that were cocultured with either UVA–treated normal human keratinocyte or normal human fibroblasts s (i.e., the targets) exhibited a consistent time-dependent increase in dichlorofluorescein (DCF) fluorescence (Figure 2b). In contrast, bystander melanocytes cocultured with unirradiated cells led to no appreciable induction of DCF fluorescence.

Experiments were then performed using all combinations of target and bystander populations to determine which cell types are receptive to bystander signaling and which cell types are efficient at generating signal when treated with UVA. Table 1 shows the corrected DCF fluorescence signal in the bystander wells after 90 minutes of coculture with

**Table 1. Cell–cell UVA bystander stress signaling**

| | UVA target (ROS donor) | | | |
|---|---|---|---|---|
| | Keratinocyte | Melanocyte | Fibroblast | Mean "recipient" index[1] |
| *Bystander (ROS recipient)* | | | | |
| Keratinocyte | 20.4[2] ± 5.4 (3)[3] | 13.5 ± 0.9 (4) | 0 ± 6.4 (3) | 11.3 |
| Melanocyte | 49.0 ± 0.5 (8) | 15.4 ± 3.5 (5) | 29.5 ± 6.7 (4) | 31.3 |
| Fibroblast | 31.3 ± 1.4 (6) | 0 ± 7.2 (4) | 11.7 ± 4.7 (4) | 14.3 |
| Mean ''donor'' index[4] | 33.6 | 9.6 | 13.7 | |

Abbreviations: DCF, dichlorofluorescein; $H_2O_2$, hydrogen peroxide; ROS, reactive oxygen species.

[1]Mean corrected stress experienced by a single recipient cell type (i.e., average of row values).

[2]To compare across experiments, we corrected UVA bystander stress to maximum stress observed with 200 μM $H_2O_2 = 100 \times (DCF_{10J} - DCF_{0J})/DCF_{H2O2}$. Thus, these values represent fluorescence normalized to unirradiated cells and adjusted to $H_2O_2$.

[3]Number of experiments shown within parentheses.

[4]Mean corrected stress among all recipients because of specific donor cell type (i.e., average of column values).

UVA–illuminated target populations. As an ROS donor, keratinocytes appeared to generate the greatest amount of bystander stress upon UVA illumination. The greatest effect was in fact observed when keratinocytes and melanocytes were paired as target and bystander (corrected stress = 49.0 ± 0.5), respectively. Similar results were obtained with two different sources of primary melanocytes. Overall, melanocytes had the highest mean ''recipient'' index compared with other cells (32.5 vs. 11.3 and 14.3), suggesting that pigment cells experience the greatest bystander stress irrespective of which target cell type was treated with UVA. Interestingly, melanocytes were the least effective donors of stress signaling when irradiated as targets, consistent with the lower response upon direct UVA exposure (Figure 1). Keratinocytes appeared to be most resistant to bystander stress, while fibroblasts showed intermediate efficiencies as both donor and recipient.

We next performed experiments to gain greater insight into the nature and extent of the signaling event. When the target fibroblasts were pre-treated with either extracellular catalase (a $H_2O_2$ scavenger) or diphenylene iodinium (an NADPH oxidase inhibitor), complete abrogation of DCF fluorescence in the melanocytes was observed (Figure 3a). These results document intercellular transmission of $H_2O_2$, and possibly other species, to bystander melanocytes upon UVA irradiation of cocultured fibroblasts. To approximate the level of oxidative signaling, we generated a standard curve of DCF fluorescence based on amounts of direct $H_2O_2$ exposure (Figure 3b). As the overall normalized melanocyte bystander DCF fluorescence (not corrected to $H_2O_2$; $N = 16$ bystander determinations) was 23.02 ± 9.3, it appears that bystander oxidative stress approaches approximately 40 μM peroxide equivalents of ROS. Furthermore, as shown in Figure 3c, both direct UVA and bystander stress signaling caused DNA damage as measured by the comet assay.

Lastly, as p53 has been shown to mitigate ROS in melanocytes, we next determined if upregulation of p53 can attenuate the UVA-mediated bystander stress. To activate p53, we used the MDM2 antagonist nutlin-3, which is known to interrupt p53-MDM2 binding and to rescue p53 from proteasomal condemnation. As shown in Supplementary Figure S2 online, pre-treatment of bystander melanocytes with nutlin-3, an MDM2 antagonist, increased cellular levels of p53 in NHMs. Strikingly, with induction of p53, there was a complete abrogation of bystander oxidative signaling in the melanocytes when cocultured with UVA–targeted fibroblasts (Figure 4).

## DISCUSSION

These studies make several important observations toward our understanding of UVA effects. A somewhat unexpected finding was that melanocytes were more resistant to direct UVA oxidative stress and the least efficient generator of bystander signaling while they were also paradoxically the most vulnerable recipients of bystander stress (Figure 5). The level of oxidative stress experienced by melanocytes within the epidermis may thus be profound considering every melanocyte is embedded within a matrix of ~36 keratinocytes (Seiberg, 2001) and deeper fibroblasts. Using approximations from our system, we estimate that the amount of bystander stress experienced by melanocytes is roughly equivalent to 40 μM $H_2O_2$ equivalents. This is likely an underestimation as the two-compartment model reflects oxidative flux diluted into a chamber filled with 500 μl of media. The cell–cell contiguity that actually exists *in vivo* would be significantly greater than the detectable amount *in vitro*. Furthermore, as human skin is stratified with keratinocytes resting on top of the melanocytes, the most active ROS ''donors'' are also the cells (i.e., keratinocytes) that come into primary contact with incoming UVA. Thus, one important biological implication of our findings is that a profound flux of near-neighbor ROS envelopes each melanocyte with every UVA exposure.

Our data suggest that stress from direct UVA exposure may be reduced in melanocytes because of melanin. There is a recent report that hypopigmented melanocytes from the *slaty* mouse (*Dct* mutation) exhibit heightened oxidative sensitivity to UVA irradiation (Wan *et al.*, 2009), which is consistent with our finding, although our melan-c2J melanocytes are completely devoid of both eumelanin and pheomelanin due to a homozygous *Tyr* mutation (Bennett *et al.*, 1989). An

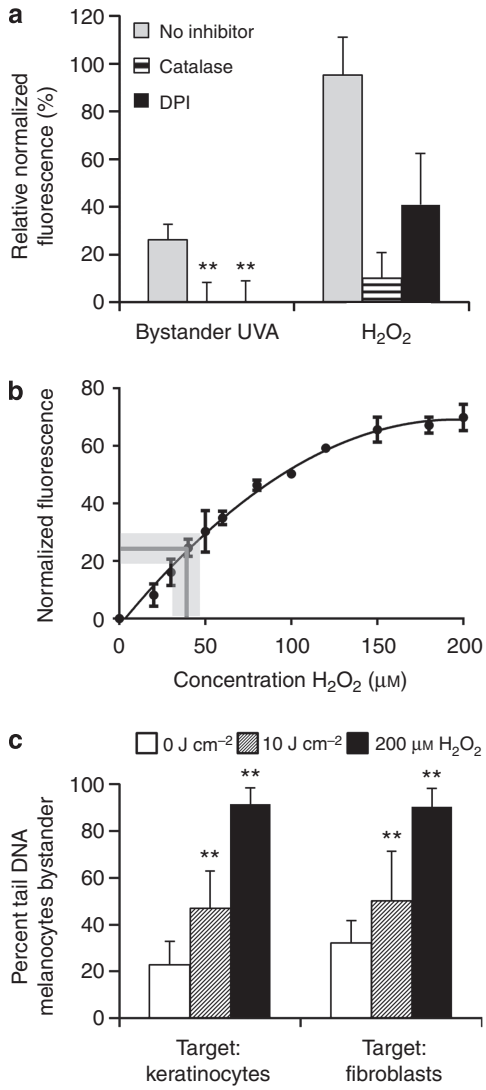


**Figure 3. Characterization of intercellular signaling.** (**a**) Loss of UVA bystander stress signaling after pre-treatment with either 50 U ml$^{-1}$ catalase or 1 μM diphenyleneiodonium iodinium (DPI). To compare across independent experiments, the normalized bystander dichlorofluorescein (DCF) fluorescence was expressed a relative percentage of the $H_2O_2$ control fluorescence (relative normalized fluorescence). Error bars indicate average of three experiments. (**b**) $H_2O_2$ (varying concentrations indicated on *x*-axis) was directly added to primary human melanocytes and the DCF was measured at 90 minutes. This time point was chosen as the UVA bystander experiments were also performed at 90 minutes. In aggregate, the level of UVA-mediated bystander stress experienced by melanocytes lies within the gray region ($N = 16$ independent bystander determinations), which corresponds to approximately the same amount of normalized fluorescence from exposure to 40 μM $H_2O_2$. (**c**) Significant increases in bystander melanocyte tail DNA after incubation of melanocytes with keratinocytes or fibroblasts that have been directly irradiated with UVA (10 J cm$^{-2}$) or directly exposed to $H_2O_2$ (200 μM). $^{**}P < 0.001$.

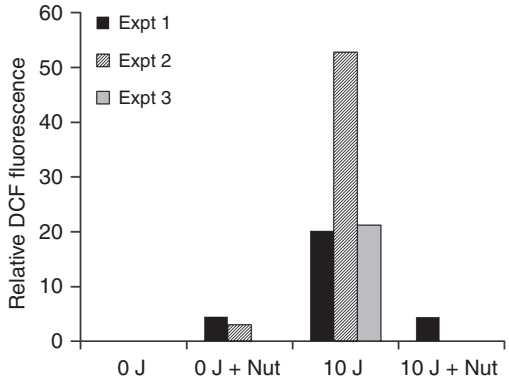


**Figure 4. Induction of p53 protects against UVA-mediated bystander stress.** Bystander primary human melanocytes were pre-treated with 5 μM nutlin-3 (Nut) for 12 hours and coincubated with UVA-irradiated target fibroblasts. The dichlorofluorescein (DCF) fluorescence (relative to unirradiated, DMSO control at 90-minute reading) is shown. All three experiments demonstrated consistent induction of bystander stress, which was uniformly abrogated with Nut pre-treatment.

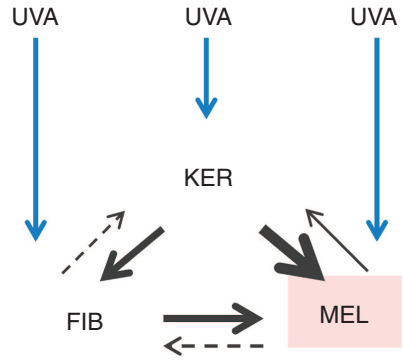


**Figure 5. Bystander model in skin.** Diagram illustrating the level of bystander stress signaling between the three skin cell types (derived from Table 1). The thicknesses of the arrows correlate with level of stress induction. Keratinocytes (KER) communicate the most significant stress upon UVA exposure, whereas melanocytes (MEL) are relatively inefficient at signaling stress to other cell types. However, both fibroblasts (FIB) and keratinocytes elicit substantial stress in melanocytes.

earlier study also showed the UVA induced more membrane permeability and lipid peroxides in unpigmented melanocytes (i.e., melan-c) compared with pigmented ones (i.e., melan-a) and more ROS in fibroblasts compared with melanocytes (Kvam and Dahle, 2003). It should also be noted, however, that NHMs have been reported to maintain higher ROS levels compared with fibroblasts, possibly due to its melanin content (Jenkins and Grossman, 2013). On the other hand, Wang *et al.* (2010) recently examined DNA photoproducts in the context of UVA irradiation and found that UVA exposure caused more oxidative DNA damage in human melanocytes compared with normal skin fibroblasts, possibly due to melanin interference with DNA repair (Wang *et al.* 2010). It should also be noted that the Wang study used maximum UVA doses of 5-fold lower than used here for our study. Thus, the cells would have been subjected to a relatively low degree of insult where bystander effects contribute to a large degree. Even in a situation where all cells are irradiated, an ''internal bystander'' effect occurs where cell signaling amplifies the stress. We have previously shown this effect to be quite marked in the case of photosensitized oxidative stress (Rubio *et al.*, 2009) where the internal bystander effect is considerable in a 2D cell population. Thus, it is possible that melanin may have

heterogeneous effects and may simultaneously absorb UVA photons, inhibit DNA repair and enhance oxidative DNA damage. Furthermore, innate differences between melanocytes and other cells independent of pigmentation may also exist. These interactions underscore the complex and unelucidated relationship between cell type, pigmentation, oxidative stress, and DNA repair.

The nature of the oxidative signaling is still under investigation. Treatment of the intercellular content with catalase appears to fully abrogate the bystander effect, thereby suggesting that $H_2O_2$ or an $H_2O_2$–like agent is the predominant signaling molecule. There is also evidence that p53 has a role in attenuating UV–induced oxidative stress. Kadekaro *et al.* (2012) reported that combined UVA + UVB irradiation of primary human melanocytes is associated with a marked increase in oxidative DNA damage that can be mitigated by p53 that is induced by either α-melanocyte-stimulating hormone or nutlin-3 (Kadekaro *et al.*, 2012). Our results are consonant with these findings. Although ongoing studies are underway to characterize mechanistic details, p53 does appear to be an important homeostatic regulator of UVA–mediated stress at least in melanocytes.

There are several limitations to our studies. The flux of oxidative species between human epidermal cells *in vivo* may be different than the levels calculated *in vitro* in our two-chamber model. The question of the likelihood of bystander effects being seen at distance in the tissue was previously studied using ionizing radiation microbeam irradiation, where scattering is negligible and targeted and bystander cells are easily identified (Belyakov *et al.*, 2005; Sedelnikova *et al.*, 2007). Using this approach in total skin constructs, it was clearly shown that DNA damage could be observed in bystander cells at distances of millimeters from the border of the targeted region. Thus, although the 2D system has its limitations, it is reasonable to expect the type of bystander responses observed to be recapitulated in living tissue. An additional limitation is that melanosome transfer between melanocytes and keratinocytes *in vivo* may attenuate direct UVA exposure within the epidermis and thus mitigate bystander stress signaling. Both of these challenges will require more faithful 3D organotypic systems and/or *in vivo* measurements, which are ongoing areas of investigation and technical development.

The public health implications cannot be understated. First, indoor sunlamps, which predominantly emit UVA, have been touted as ''safe'' given their relatively lower levels of genotoxic UVB (Autier *et al.*, 2011). Our studies used a fluence of $10\,J\,cm^{-2}$ delivered at an irradiance of $10.5\,mW\,cm^{-2}$ for a duration of approximately 16 minutes. Literature reports of sunbed characteristics cite irradiances of $\sim 20\,mW\,cm^{-2}$ UVA delivered to the skin for a fluence of $24–36\,J\,cm^{-2}$ in a typical 20–30 minutes session—a level higher than that delivered in our experiments (McGinley *et al.*, 1998). In addition, the UVA irradiance at the Earth's surface in several cities across the United States has been estimated to be around $2.5\,mW\,cm^{-2}$; hence, the fluence of $10\,J\,cm^{-2}$ is equivalent to just over an hour's exposure under physiological conditions (Grant and Slusser, 2005). Recent whole animal studies have also shown that UVA is fully competent to induce melanomas in a melanin-dependent manner and that UVA preferentially creates 8-oxo-7,8-dihydro-2′-deoxyguanosine, which results from ROS (Noonan *et al.*, 2012). Thus, our study broadens the scope of UVA–induced sun damage and perhaps speaks to the long-term effects of sunlamps. Furthermore, as sunlight itself is 90–98% UVA (Autier *et al.*, 2011), sunscreens that solely absorb UVB without significantly attenuating UVA will have little impact on the levels of diffusible ROS generated by keratinocytes upon solar UVA exposure.

In summary, the emerging connection between UVA and melanoma risk has uncovered fundamental gaps in our understanding of UVA photocarcinogenesis. Our findings suggest that near-neighbor cells within the cutaneous community are vulnerable to significant levels of bystander stress and that a dynamic flux of ROS may be created during intense whole-body UVA irradiation whether intentionally from sunlamp use or unintentionally from poor UVA sun protection.

## MATERIALS AND METHODS

### Cell culture and compounds

Primary neonatal human keratinocyes were a gift from Dr James Reinwald at Brigham and Women's Hospital (Boston, MA) and were cultured in 75 ml flasks with keratinocyte serum-free medium (Invitrogen, Grand Island, NY) supplemented with bovine pituitary extract (final concentration of $25\,\mu g\,ml^{-1}$), epidermal growth factor (final concentration of $0.2\,ng\,ml^{-1}$), 0.3 mM $CaCl_2$, and 10% penicillin/streptomycin. Medium was exchanged every 2 days and keratinocytes were grown (passages 3–13) in the flask until 70% confluent. Human dermal fibroblasts (adult) were purchased from Life Technologies (Grand Island, NY) and cultured in 10 cm diameter plates with Medium 106 (M-106-500; Life Technologies) supplemented with low serum growth supplement (5 ml in 500 ml of media) (Life Technologies) and 10% penicillin/streptomycin. Medium was exchanged every 2–3 days and fibroblasts were grown in the plate until 90% confluent. Two sources of primary human epidermal melanocytes were used: adult, lightly pigmented (HEMa-LP) melanocytes were purchased from Life Technologies and neonatal foreskin melanocytes were obtained from Dr Mark Pittelkow (Mayo Clinic, Rochester, MN). These melanocytes were cultured in 10 cm diameter plates in Medium 254 (Life Technologies) containing human melanocyte growth supplement (5 ml in 500 ml of media; Life Technologies) and 10% penicillin/streptomycin. Medium was exchanged every 2–3 days and melanocytes were grown in the plate until 90% confluent. The primary human fibroblasts and keratinocytes used were less than passage 12. Primary human melanocytes with slightly different passages were used with similar results, although no primary human melanocytes after passage 14 were used. Melan-a (nonagouti/black (*a/a*), C57/BL6 background) and melan-c (albino ($Tyr^{c-2J}/Tyr^{c-2J}$), C57/BL6 background) cells were obtained through a collaboration with Dr Elena V. Sviderskaya.

Twenty-four hours before the experiment, the medium was removed and cells were trypsinized (0.25% trypsin/EDTA) and replated in either 6-well plates (Becton-Dickinson; Franklin Lakes, NJ, BD-353504) or companion transwell inserts (BD-353104). Wells were plated as 80,000 cells per well and inserts were plated at 40,000 cells per well. Irradiated cells in the inserts comprise the target

population, whereas unirradiated cells in the wells comprise the ''bystander'' population.

Both diphenyleneiodonium chloride and catalase were purchased from (Sigma, St. Louis, MO) and used at a final concentration of 1 μM and 50 U ml$^{-1}$, respectively.

### UVA treatment

Immediately before treatment, the medium in the inserts was removed and replaced with 300 μl of Hank's balanced salt solution. Inserts were irradiated from above with a UVP Blak–Ray UV lamp (Ted Pella, Reading CA) for a period of time sufficient to deliver a total fluence of 10 J cm$^{-2}$ to the sample. The typical irradiance was ~11 mW cm$^{-2}$, which required a total of about 15 minutes illumination. The spectral output of the lamp was measured using a calibrated SP-01 spectroradiometer (Luzchem, Ottawa, ON, Canada) and is shown in the Supplementary Figure S1. The output has a maximum wavelength around 365 nm and is entirely in the UVA spectrum with negligible UVB contribution.

*Coculture.* During UVA illumination of cells in the insert, the medium was removed from a partner well containing a non-illuminated cell population and replaced with 500 μl of Hank's balanced salt solution (Life Technologies). On completion of UVA treatment, the insert was placed in the companion well, designated as time zero. The insert has a semipermeable membrane interface with 1 μm pore size that allows exchange of small molecules but cells are kept separate. Coculture was then performed in a humidified incubator at 37 °C with 5% $CO_2$. This is shown schematically in Figure 2a.

*Cell viability.* Viability was measured in cell populations by flow cytometry using either Biotium Viability/Cytotoxicity Assay kit. Viability was also determined by confocal microscopy using the Live/Dead assay kit (catalog no.: MP-03224; Life Technologies). Cells ~70–80% confluent were irradiated in a 35 mm plate, incubated for 4 hours, and then removed by trypsinization, normalized in medium, washed in phosphate-buffered saline, and resuspended in Opti-MEM medium (Life Technologies) for 30 minutes before insertion into a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA). For confocal imaging, after irradiation, the cells were incubated for 4 hours in Opti-MEM medium and the Live/Dead assay dyes were added and imaged.

*Measurement of oxidative stress.* Immediately following illumination of the insert, a further 200 μl of Hank's balanced salt solution containing 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA; Life Technologies) was added to the insert to give a total volume of 500 μl in the insert with a final CM-$H_2$DCFDA concentration of 1.7 μM. At the same time, the medium in the companion well was removed and replaced with 500 μL of Hank's balanced salt solution containing CM-$H_2$DCFDA at a concentration of 1.7 μM. The insert was then placed in the companion well at time zero and placed in the incubator at 37 °C. On oxidation, the non-fluorescent CM-$H_2$DCFDA is converted to the fluorescent product, DCF. At 15 minutes durations, the well plate was taken to the plate reader, the inserts removed, and the fluorescence intensity in each well was measured using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) with excitation and detection at 488 and 525 nm, respectively.

### Comet assay

The assay was performed using the Trevigen Comet Assay kit (catalog no.: 4250-050-K). Ten microliters of cell suspension (~$10^5$ cells) was added to 100 μl of low-melting point agarose and 100 ml aliquot was then dropped onto a precoated slide. The slides were placed at 4 °C in the dark for 10 minutes and then immersed in the prechilled lysis solution provided in the kit and incubated for 1 hour at 4 °C. The excess buffer was drained and the slides immersed in freshly prepared alkaline unwinding solution (300 mM NaOH, 1 mM EDTA, pH = 13) for 1 hour at room temperature in the dark. The slides were then placed in an electrophoresis tank, with prechilled alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH >13). Electrophoresis was carried out for 20 minutes at 24 V. The excess solution was drained and the slides were rinsed two times in deionized water for 5 minutes and then in 70% ethanol for 5 minutes. The slides were dried at <45 °C for 10–15 minutes. One hundred microliters of diluted Syber gold (Stock 10,000X; Invitrogen; S-11494) was placed on the dried agarose and the slides were kept at 4 °C for 5 minutes. Excess solution was removed and the slides were allowed to dry at room temperature. Slides were viewed using a confocal microscope (Olympus Fluoview FV1000, Center Valley, PA; Ex/Em 495/537 nm). For quantitative analysis, 50 randomly chosen nuclei were considered and comet scoring performed using the Image J comet assay plug in. (www.med.unc.edu/microscopy/resources/imagej-plugins-and-macros/comet-assay)

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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